

### REMARKS

Favorable reconsideration of the subject application is respectfully requested in view of the following remarks. By this amendment, claims 1 and 3-19 are canceled. This amendment is not to be construed as acquiescence to any rejection and is made without prejudice to prosecution of any subject matter modified by the amendment in a related divisional, continuation, or continuation-in-part application.

#### Approval of Petition to Withdraw Holding of Abandonment

Applicants thank the Examiner for noting that the Petition to Withdraw Holding of Abandonment, filed January 9, 2004, was approved.

#### Rejection Under 35 U.S.C. § 102(b)

Claims 21-25 stand rejected under 35 U.S.C. § 102(b), as allegedly anticipated by Brinkman *et al.* More specifically, the Examiner asserts that Brinkman *et al.* is referenced in NCBI entry NP\_065144.1 (XAGE-1), which provides a polypeptide sequence of XAGE-1, having a 98% overall homology to SEQ ID NO:809. Based upon this reference, the Examiner appears to incorrectly draw the conclusion that Brinkman *et al.* discloses the polypeptide sequence provided in NCBI entry NP\_065144.1 (XAGE-1).

Applicants traverse this basis of rejection and maintain that Brinkman *et al.* fails to describe the claimed invention, and, therefore, the Action has not established a *prima facie* case of anticipation.

As fully explained in the previous Amendment submitted August 21, 2003, Brinkman *et al.* absolutely fails to describe the claimed polypeptides, namely variants and fragments of SEQ ID NO:809. Contrary to the Examiner's assertion, Applicants submit that Brinkman *et al.* does not disclose the polypeptide sequence of XAGE-1. Indeed, Brinkman *et al.* explicitly state that they could not determine and, consequently, do not disclose the polypeptide sequence of XAGE-1. "Because of the uncertainty with translation, this gene was omitted from Fig. 3 [alignment of the deduced amino acid sequences of XAGE-2 and XAGE-3 with a typical GAGE gene]" (page 1447, column 2, lines 16-18). Thus, while Brinkman *et al.* describes the

identification of partial polynucleotide sequences (ESTs) corresponding to XAGE-1, it absolutely fails to identify or describe the XAGE-1 polypeptide sequence. Nor is the polypeptide sequence implicitly disclosed by Brinkman *et al.* based upon the identification of partial polynucleotide sequences, since the authors explicitly concede that the XAGE-1 polypeptide sequence could not be determined based upon the identified polynucleotide sequences. Since Brinkman *et al.* absolutely fails to provide the polypeptide sequence of XAGE-1, it clearly cannot anticipate the presently claimed polypeptide sequences.

Given that Brinkman *et al.* fails to provide the polypeptide sequence of XAGE-1, the Examiner has attempted to work backwards from Applicants' sequence (SEQ ID NO:809) to establish some basis on which to rely in his assertion that Brinkman *et al.* discloses the XAGE-1 polypeptide sequence and, therefore, anticipates the presently claimed invention. To this end, the Examiner searched the NCBI database to identify accession number NP\_065144, which provides the XAGE-1 polypeptide sequence and lists Brinkman *et al.* in its list of related references. However, a review of the history of NCBI entry NP\_065144 unequivocally states that "accession NP\_065144.1 was first seen at NCBI on Sept 5 2000." Accordingly, this accession number was not publicly available until at least September 5, 2000, which is after the priority date afforded the instant application, which is at least as early as April 27, 2000, and, therefore, does not qualify as prior art under Section 102. For the Examiner's convenience, a copy of the Revision History for NP\_065144, including this statement, is attached.

Furthermore, the fact that Brinkman *et al.* is provided in a list of publications related to the sequence listing in no way establishes that Brinkman *et al.* itself provides the listed sequence. In fact, as described in detail above, careful review of Brinkman *et al.* clearly reveals that Brinkman *et al.* fails to disclose the polypeptide sequence of XAGE-1 or the presently claimed sequences. In addition, Applicants emphasize that the comment provided directly below the listing of references in NCBI entry NP\_065144.1 clearly states that the reference sequence disclosed therein was derived from AF251237.1, which is a full length cDNA sequence of XAGE-1. However, the AF251237.1 sequence was not seen by NCBI until August 3, 2000, according to the Revision History for AF251237.1, a copy of which is attached for the Examiner's convenience. This, of course, makes perfect sense, since the full length XAGE-1

cDNA and predicted open reading frame were not identified until the work of Liu *et al.*, described in Cancer Research 60: 4752-4755 (September 1, 2000)(copy attached), which was also published after the priority date of the instant application.

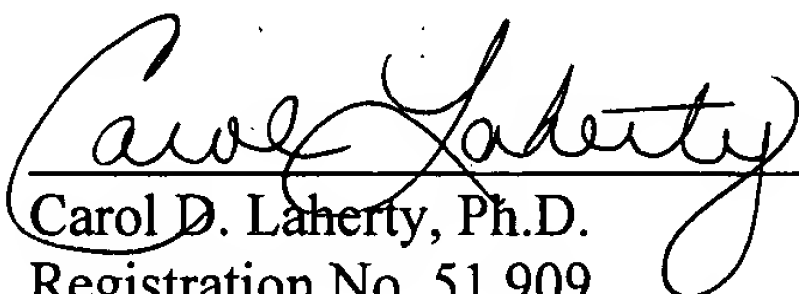
To summarize, Applicants submit that Brinkman *et al.* fails to anticipate the presently claimed invention, since Brinkman *et al.* fails to disclose the claimed sequences (and even explicitly admits that the authors were not in possession of the claimed polypeptides or sequences). In addition, Applicants submit that the XAGE-1 polypeptide sequence that the Examiner is attempting to cite against the present claims was not publicly available until at least September 5, 2000, according to NCBI records, which is after the priority date of the instant application. Accordingly, the instant claims are not anticipated by Brinkman *et al.* or the NCBI XAGE-1 sequence. Applicants respectfully request that the Examiner reconsider and withdraw this basis of rejection.

The Director is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Applicants submit that all of the claims remaining in the application are clearly allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

Respectfully submitted,

SEED Intellectual Property Law Group PLLC

  
Carol D. Laherty, Ph.D.  
Registration No. 51,909

CDL:jto

Enclosures:

Postcard

Copy of Revision History for NP\_065144

Copy of Revision History for AF251237.1

Copy of Liu *et al.*, Cancer Research 60, 4752-4755

701 Fifth Avenue, Suite 6300  
Seattle, Washington 98104-7092  
Phone: (206) 622-4900  
Fax: (206) 682-6031

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9966899	1	<a href="#">Dec 21 2003 9:43 AM</a>	Live	<input checked="" type="radio"/>	<input type="radio"/>
9966899	1	<a href="#">Oct 5 2003 12:40 AM</a>	Dead	<input type="radio"/>	<input checked="" type="radio"/>
9966899	1	<a href="#">Sep 6 2003 6:54 PM</a>	Dead	<input type="radio"/>	<input type="radio"/>
9966899	1	<a href="#">Apr 6 2003 9:56 AM</a>	Dead	<input type="radio"/>	<input type="radio"/>
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9966899	1	<a href="#">Sep 5 2000 8:43 AM</a>	Dead	<input type="radio"/>	<input type="radio"/>

Accession NP\_065144.1 was first seen at NCBI on Sep 5 2000 8:43 AM

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9885324	1	Aug 23 2000 12:04 AM	Live

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**XAGE-1, A New Gene That Is Frequently Expressed in Ewing's Sarcoma<sup>1</sup>**Xiu Fen Liu, Lee J. Helman, Choy Yeung, Tapan K. Bera, Byungkook Lee, and Ira Pastan<sup>2</sup>

Laboratory of Molecular Biology, Division of Basic Sciences, NIH, National Cancer Institute, Bethesda, Maryland 20892-4255 [X. F. L., T. K. B., B. L., I. P.], and Pediatric Oncology Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, Maryland 20892 [L. J. H., C. Y.]

**Abstract**

Our previous expressed sequence tag database analysis indicates that *XAGE-1* is frequently found in Ewing's sarcoma and alveolar rhabdomyosarcoma (U. Brinkmann *et al.*, *Cancer Res.*, 59: 1445-1448, 1999). Using Northern blots and RNA dot blots, we have now found that *XAGE-1* is highly expressed in normal testis, in seven of eight Ewing's cell lines, in four of nine Ewing's sarcoma patient samples, and in one of one alveolar rhabdomyosarcoma patient sample. The gene is located on the X chromosome. The full-length cDNA contains 611 bp and predicts a protein of *M<sub>r</sub>* 16,300 with a potential transmembrane domain at the NH<sub>2</sub> terminus. *XAGE-1* shares homology with GAGE/PAGE proteins in the COOH-terminal end. These findings could be valuable for cancer diagnosis and cancer immunotherapy.

**Introduction**

Large numbers of ESTs<sup>3</sup> have been cloned from various tissues and cancers (1, 2). Each cDNA clone or EST sequence is generated from a single transcript. The frequency and distribution of the many different transcripts in a given tissue depends on the level of gene expression. Therefore, a particular gene expression pattern can be frequently predicted by analysis of the frequency and specificity of various EST sequences. We previously reported a computer screening strategy that identified genes that are preferentially expressed in prostate or prostate tumors (3-5). From this screen, numerous genes were identified including a novel gene, *PAGE4* (previously named *PAGE1*) (6), and a set of *XAGE* genes (7), which are related to the GAGE/MAGE family of melanoma associated CT antigens.

CT antigens are a distinct class of differentiation antigens that have a restricted pattern of expression in normal tissues (8-10). CT antigens are currently viewed as attractive candidates for cancer vaccines. Some thoroughly studied CT antigens are MAGE, BAGE, GAGE, and LAGE/NY-ESO-1 (9-17). These genes are primarily expressed in the primitive germ cells, spermatogonia, in the normal testis. Malignant transformation is often associated with activation or derepression of silent CT genes, and this results in the expression of CT antigens in a variable proportion of a wide range of human tumors. Recently, several additional members were added to the CT antigen family. These include various PAGEs, PRAME, SSX, SCP-1, CT7, MAGEC1, and MAGED1 (6, 11, 18-22). Identification of new CT antigens or new family members is still being actively pursued in the cancer research field.

Previously, we identified three related genes that we termed *XAGEs* by homology walking using the dbEST database (7). ESTs of the

*XAGE* group were found in cDNA libraries from testis, Ewing's sarcoma, alveolar rhabdomyosarcoma, fetal tissues, and germ cell tumors by database analysis. In this study, we describe the isolation of a full-length cDNA of *XAGE-1* and analysis of the expression of the *XAGE-1* gene. We found that the gene is highly expressed in normal testis, with aberrant expression in sarcomas of various types. Although *XAGE-1* did not show overall homology with any polypeptide recorded in the data bank, it has strong homology with members of GAGE/PAGE family in the COOH terminus of the predicted protein, indicating that *XAGE-1* belongs to a new family of CT antigens.

**Materials and Methods**

**Tissues and Cell Lines.** Ewing's tumor tissue was obtained from frozen specimens obtained from patients treated at the National Cancer Institute. Rhabdomyosarcoma tumor tissue was obtained from the Cooperative Human Tissue Network, Children's Cancer Group. All alveolar rhabdomyosarcoma tumor specimens were found to express the PAX-3-FKHR fusion transcript by RT-PCR.<sup>4</sup> Osteosarcoma cell lines were obtained from the American Type Culture Collection. Ewing's sarcoma cell lines RD-ES, TC-32, TC-71, and 5838 have been described previously, and all contain EWS-FLI-1 fusion transcripts (23).<sup>4</sup> LD, LG, JM, and SB are cell lines established in our laboratories (by L. J. H.) directly from tumor specimens. The cell line JM does not express an EWS-ETS fusion transcript.

**Northern Blots and RNA Dot Blot.** RNA was extracted either from tumor tissue using Trizol (Life Technologies, Inc.) or from cell lines using RNeasy (Qiagen). Total RNA (20 µg) was used for Northern blot analysis of sarcoma tumors. The multiple tissue mRNA dot blot and the normal tissue Northern blot were purchased from Clontech (Palo Alto, CA). The 450-bp probe used for hybridization was generated from EST clone af89d01.s1 by digestion with *EcoRI* and *NorI*. The hybridization was conducted as follows: the RNA-containing membranes were blocked for 3 h at 45°C in hybridization solution. Probes labeled with <sup>32</sup>P either by random primer extension or by end labeling (Lofstrand Labs Limited) were added to the membrane and hybridized for 15 h at 45°C. Membranes were washed twice with 2X SSC/0.1% SDS at room temperature and twice with 0.1X SSC/0.1% SDS at 65°C. The membranes were exposed to X-ray film for 1-2 days before development.

The Southern blot of human chromosomes (Oncor, Gaithersburg, MD) was conducted using the same probe and the same hybridization conditions used for Northern blot.

RT-PCR was performed on cDNA from 24 different human tissues using human rapid-scan gene expression panels (Origene, Inc., Rockville, MD). The thermocycling protocol was initial denaturation at 94°C for 3 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 3 min. The PCR reactions were analyzed on agarose gels, and specific products were cloned into TA vectors (Invitrogen) and sequenced on an automated capillary sequencer using Perkin-Elmer's dRhodamine terminator cycle sequencing kit (Perkin-Elmer Applied System).

The primers used were as follows: (a) xa-1, 5'-CAGCTTGCTTCATTTA-AACTTGTGGTTGC-3'; (b) xa-2, 5'-TCCCAGGAGCCCAGTAATGGAGA-3'; (c) xa-8, 5'-ACCTGGGAAGGAGCATAGGA-3'; and (d) xa-10, 5'-CTT-TATTGAGATAGTTTAAGTCAAATATCTAA-3'. The oligonucleotides were synthesized by Sigma-Genosys.

<sup>4</sup> Unpublished observations.

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<sup>2</sup> To whom requests for reprints should be addressed, at Laboratory of Molecular Biology, Division of Basic Sciences, NIH, National Cancer Institute, Building 37, Room 4E16, 37 Convent Drive 4255, Bethesda, MD 20892-4255. Phone: (301) 496-4797; Fax: (301) 402-1344; E-mail: pasta@helix.nih.gov.

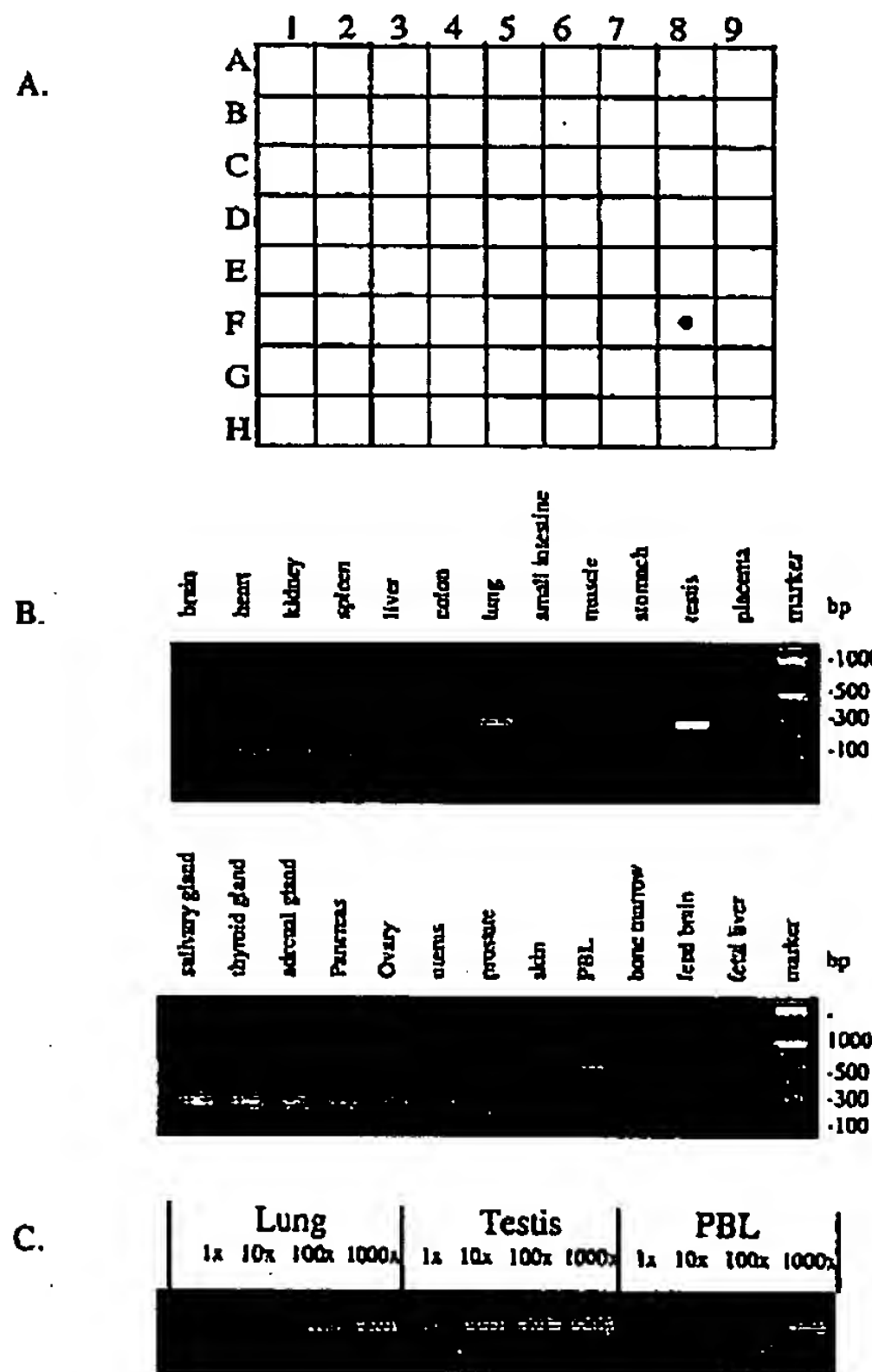
<sup>3</sup> The abbreviations used are: EST, expressed sequence tag; CT, cancer-testis; RT-PCR, reverse transcription-PCR; PBL, peripheral blood leukocyte.



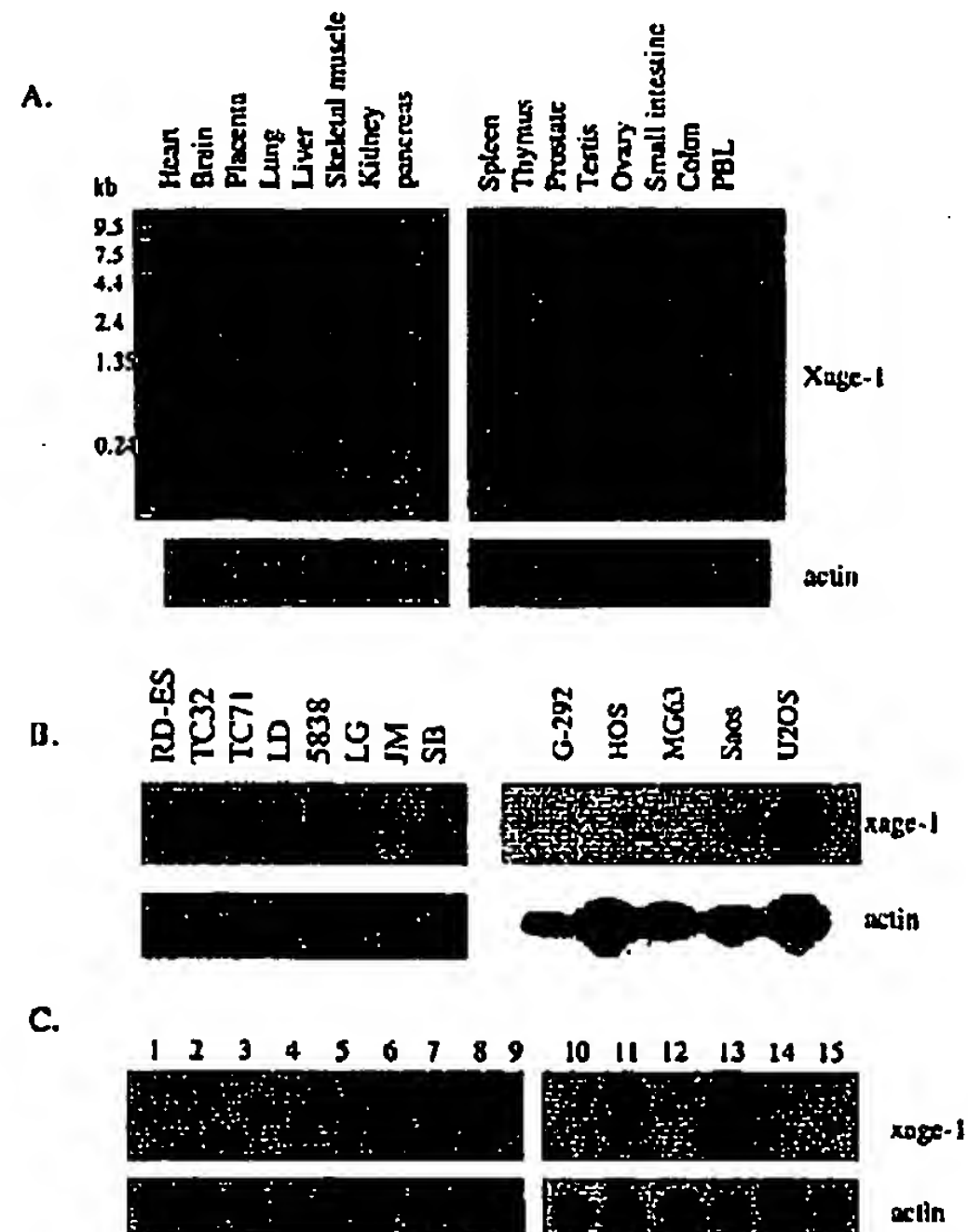
## Results

**Restricted Expression in Normal Tissues.** To determine the relative expression of *XAGE-1* mRNA in different tissues and tumors, we first conducted a mRNA dot blot (Clontech) analysis using a full insert of EST af89d01.s1 as a labeling probe. Among the 61 different samples of normal tissues and 7 fetal tissues including lung, brain, liver, heart, and spleen, the expression of *XAGE-1* was detected only in testis (Fig. 1A). This result indicates that *XAGE-1*, like other CT antigens, is present in testis.

To verify the specificity of *XAGE-1* expression, we conducted a RT-PCR analysis by using the human rapid-scan panel with primers xa-1 and xa-2. We detected a 275-bp fragment in testis among the 24 different tissues analyzed (Fig. 1B). Unexpectedly, the 275-bp frag-



**Fig. 1. Specific expression of *XAGE-1*.** A, RNA dot blot hybridization. Human multiple tissue master blot with 76 tissue-specific poly(A) RNA (Clontech) were hybridized with *XAGE-1*-specific probe. A1, whole brain; B1, cerebral cortex; C1, frontal lobe; D1, parietal lobe; E1, occipital lobe; F1, temporal lobe; G1, p.g. of cerebral cortex; H1, pons; A2, cerebellum left; B2, cerebellum right; C2, corpus callosum; D2, amygdala; E2, caudate nucleus; F2, hippocampus; G2, medulla oblongata; H2, putamen; A3, substantia; B3, nucleus accumbens; C3, thalamus; D3, pituitary gland; E3, spinal cord; A4, heart; B4, aorta; C4, atrium left; D4, atrium right; E4, ventricle left; F4, ventricle right; G4, interventricular septum; H4, apex of the heart; A5, esophagus; B5, stomach; C5, duodenum; D5, jejunum; E5, ileum; F5, ileoceum; G5, appendix; H5, colon ascending; A6, colon transverse; B6, colon descending; C6, colon rectum; A7, kidney; B7, skeletal muscle; C7, spleen; D7, thymus; E7, PBLs; F7, lymph node; G7, bone marrow; H7, trachea; A8, lung; B8, placenta; C8, bladder; D8, uterus; E8, prostate; F8, testis; G8, ovary; A9, liver; B9, pancreas; C9, adrenal gland; D9, thyroid gland; E9, salivary gland; F9, mammary gland. B, RT-PCR analysis. Twenty-four different sources of cDNA (Origene) were amplified by using primers xa-1 and xa-2 under the conditions described in "Materials and Methods." After the PCR cycles, 5  $\mu$ l of 25  $\mu$ l of the high-concentration (1000 $\times$ ) reactions were analyzed in agarose gel and visualized by staining with ethidium bromide. C, four different concentrations from lung, testis, and PBLs amplified in A were run together to compare the relative level of *XAGE-1* expression. F3-H3, D6-H6, H8, G9, and H9 are empty.



**Fig. 2. Northern blot analysis of the *XAGE-1* gene.** A, Northern blot (Clontech) of 16 different normal tissues probed separately with *xage-1* and actin probe. B, the expression of *XAGE-1* in Ewing's sarcoma cell lines (left panel) and osteosarcoma cell lines (right panel). C, the expression of *XAGE-1* in patient tumor samples. Lanes 1-9, Ewing's sarcoma patients; Lane 10, normal muscle; Lane 11, alveolar rhabdomyosarcoma; Lane 12, fetal muscle; Lanes 13-15, embryonal rhabdomyosarcoma. Total RNA was probed separately with *xage-1* probe and actin probe.

ment was also present at lower amounts in normal lung and PBLs. Extremely weak expression of *XAGE-1* was detected in bone marrow, spleen, and skin. To compare the relative level of *XAGE-1* in testis, lung, and PBLs, different dilutions of cDNA were analyzed in the same rapid-scan panel as shown in Fig. 1C. The mRNA present in testis was about 10-100 times higher than that in the lung and more than 100 times higher than that in PBLs.

Because *XAGE-1* is highly abundant in testis and is expressed at a low level in lung and PBLs, we attempted to determine the transcript size in these different tissues. Northern blot analysis was conducted using the same probe used for the RNA dot blot. As shown in Fig. 2A, a single band of 700 bp was revealed in the testis. However, no signal was detected in lung and PBLs. This result is probably due to the low level of *XAGE-1* expression in lung and PBLs because the Northern blot analysis is much less sensitive than RT-PCR in detecting the expression of *XAGE-1*. These results are consistent with the RNA dot blot analysis described above (Fig. 1A).

***XAGE-1* Expression in Ewing's Sarcoma, Rhabdomyosarcoma, and Osteosarcoma.** Analysis of the EST database predicts that *XAGE-1* is present in Ewing's sarcoma and alveolar rhabdomyosarcoma. To confirm the database prediction experimentally, we first determined whether *XAGE-1* was present in the various Ewing's cell lines by Northern blot analysis. A single band of 700 bp was detected in seven of eight cell lines (Fig. 2B). *XAGE-1* was not expressed in cell line JM, which is a mouse xenograft tumor derived from a Ewing's sarcoma that has lost the chromosome translocation t(11;22).<sup>4</sup> Cell line 5838 had an extra band with a size of 1.2 kb. This band might be due to alternate splicing or use of an alternate polyadenylation signal in the *XAGE-1* gene.

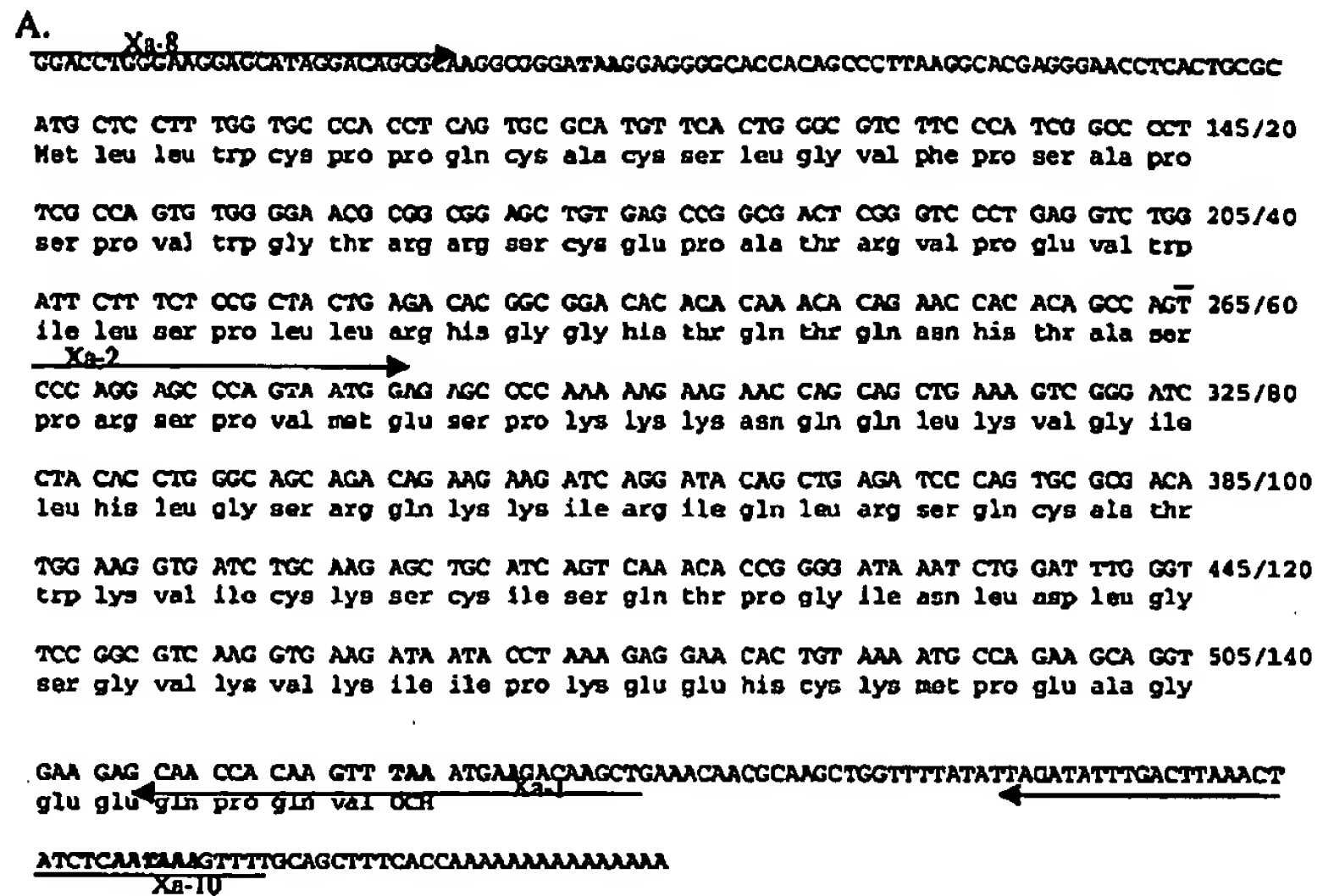
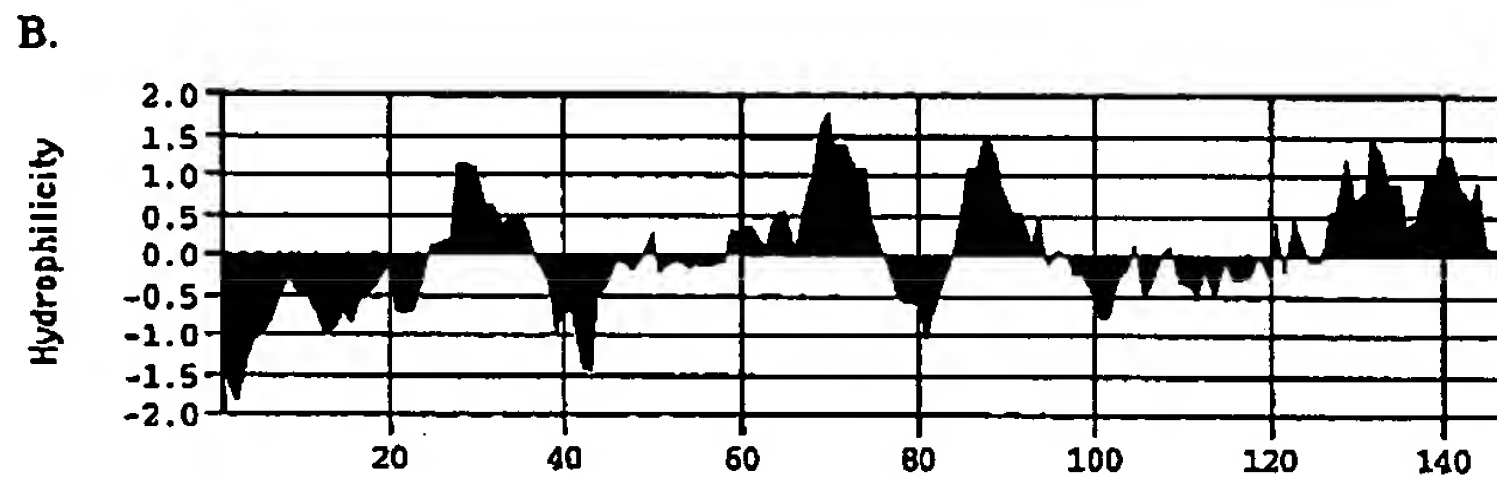


Fig. 3. Sequence of *XAGE-1*. A, the nucleotide and amino acid sequence of *XAGE-1*. The polyadenylation signal is italicized and bold. The translational stop codons are indicated in bold. Primers are showed by arrows. B, Hoop/Woods hydrophilicity plot. A potential membrane-spanning region was noticed in the NH<sub>2</sub>-terminal end.



*XAGE-1* was present in two of five osteosarcoma cell lines, with the SAOS cell line showing relatively low *XAGE-1* expression (Fig. 2B).

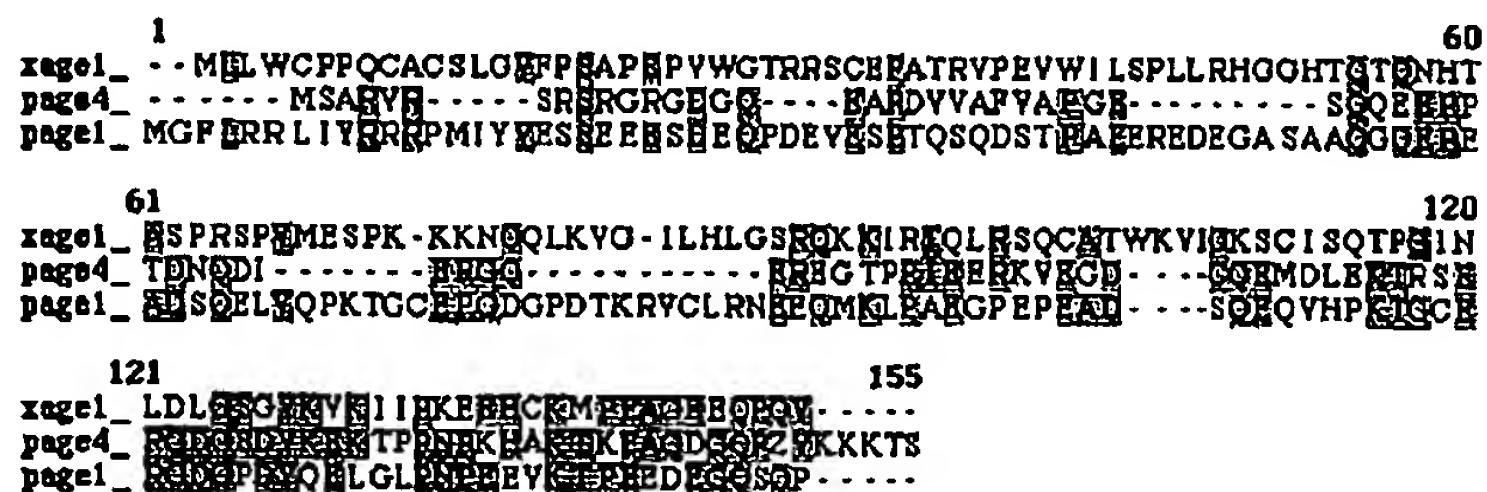
To address whether *XAGE-1* was present in human patient samples, we conducted a Northern blot hybridization analysis. Of nine patients with Ewing's sarcoma, four (patients 5–8) expressed *XAGE-1* with a single 700-bp band (Fig. 2C). Patients 1 and 5–9 expressed the *EWS-FLI-1* transcript, an indication of chromosome translocation (Ref. 24; data not shown). We noticed that *XAGE-1* was not expressed in all of the patient samples with the chromosome translocation. However, samples that did not express either an *EWS-FLI-1* or an *EWS-ERG* fusion transcript also did not express *XAGE-1* (Fig. 2C, Lanes 2–4). The correlation between the absence of *XAGE-1* expression and the absence of chromosome translocation needs further study. *XAGE-1* was also expressed in one of one patient sample with alveolar rhabdomyosarcoma and in one of three patient samples of embryonal rhabdomyosarcoma but not in the normal controls

(Fig. 2C). Together, these data indicate that *XAGE-1* is expressed in nearly half of the sarcoma patient samples.

**Chromosome Localization of *XAGE-1*.** Most of the CT antigens are localized on the X chromosome, with the exception of SCP-1, which is located on chromosome 1 (8, 20). To find where *XAGE-1* is localized, Southern blot hybridization was performed on a human chromosome blot using the same probe used for dot blot and Northern blot. We detected only one strong band on the X chromosome, and no other cross-hybridizing bands were found on the blot (data not shown). This result indicates that the *XAGE-1* gene is located on the X chromosome and that there is not a very strong homology with the other predicted *XAGE* members, *XAGE-2* and *XAGE-3*, because under stringent hybridization conditions, *XAGE-2* and *XAGE-3* were not detected.

**Full-length cDNA of *XAGE-1* and Putative Peptide Sequences.** To obtain the full-length *XAGE-1* cDNA sequence, rapid amplification of cDNA ends-PCR was performed using primers localized in the

Fig. 4. Comparison of the *XAGE-1*, *PAGE-4*, and *PAGE-1* (*GAGE9*) amino acid sequences. Conserved residues are shaded.





EST contig and total RNA from Ewing's sarcoma cell line TC-71. The longest rapid amplification of cDNA ends product contains an additional 184 nucleotides at the 5' end compared with the EST contig sequence. The correct cDNA sequence was confirmed by sequencing the PCR product from primers xa-8 and xa-10. The *XAGE-1* cDNA is 611 bp in length, excluding the poly(A) tail, and contains 438 nucleotides in the coding region, flanked by 85 bp in the 5'-untranslated region and 88 bp in the 3'-untranslated region (Fig. 3A).

The longest open reading frame indicates that the putative *XAGE-1* protein consists of 146 amino acid residues with a molecular weight of 16,300. Hydrophilicity analysis (Fig. 3B) indicates a hydrophobic sequence in the NH<sub>2</sub>-terminal end, suggesting the possibility of a transmembrane domain. Analysis of the protein sequence reveals no possible posttranslational modifications by searching GCG Lite. This putative protein did not show overall sequence homology with any peptide recorded in the data banks. However, alignment of the amino acid sequence of *XAGE-1* with PAGE4 (6) and PAGE1 [Ref. 25 (renamed GAGE9; Ref. 15)] reveals a striking homology in the COOH-terminal end of these proteins (Fig. 4), suggesting that *XAGE-1* encodes a distinct protein that could share structural or functional features with other GAGE/PAGE family members.

### Discussion

*XAGE-1* is a human X-linked gene that is strongly expressed in normal testis, Ewing's sarcoma, and alveolar rhabdomyosarcoma. It is also expressed in normal lung and PBLs, but at much lower levels. *XAGE-1* expression in lung and lymphocytes was not predicted by EST database analysis, probably because of its weak expression and the small size of lung and PBL libraries. The expression pattern of *XAGE-1* in Ewing's sarcoma, lung, PBLs, and bone marrow is interesting. At present, it is unclear in which cell type Ewing's sarcoma originates and why it metastasizes to the lung. The high level of *XAGE-1* expression in many Ewing's sarcomas suggests that the tumor could arise in cells in the bone or bone marrow that normally express *XAGE-1* or metastasize to the region where *XAGE-1* is expressed. Additional experiments using *in situ* hybridization or immunohistochemistry should provide information about this possible relationship.

Until now, there has been no report on the expression of CT antigens in Ewing's sarcoma and alveolar rhabdomyosarcoma. We found *XAGE-1* expressed in seven of eight Ewing's sarcoma cell lines, 44% (four of nine) of Ewing's sarcoma tumor specimens, and one of one alveolar rhabdomyosarcoma tumor sample. We are currently screening the expression of *XAGE-1* in other tumor cell types. Our preliminary data indicate that the *XAGE-1* gene is activated in many tumor cell lines and other tumor types. Studies on the *XAGE-1* gene may provide more information on this cancer antigen family that may be of use in the diagnosis or immunotherapy of cancer.

### Acknowledgments

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